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Synergistic toxicity of Macondo crude oil and dispersant Corexit 9500A® to the *Brachionus plicatilis* species complex (Rotifera)

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ABSTRACT

Using the marine rotifer *Brachionus plicatilis* acute toxicity tests, we estimated the toxicity of Corexit 9500A®, propylene glycol, and Macondo oil. Ratios of 1:10, 1:50 and 1:130 for Corexit 9500A®:Macondo oil mixture represent: maximum exposure concentrations, recommended ratios for deploying Corexit (1:10–1:50), 1:130 the actual dispersant:oil ratio used in the Deep Water Horizon spill. Corexit 9500A® and oil are similar in their toxicity. However, when Corexit 9500A® and oil are mixed, toxicity to *B. manjavacas* increases up to 52-fold. Extrapolating these results to the oil released by the Macondo well, suggests underestimation of increased toxicity from Corexit application. We found small differences in sensitivity among species of the *B. plicatilis* species complex, likely reflecting phylogenetic similarity. Just 2.6% of the water-accommodated fraction of oil inhibited rotifer cyst hatching by 50%, an ecologically significant result because rotifer cyst in sediments are critical resources for the recolonization of populations each Spring.

1. Introduction

The April 2010 oil spill in the Gulf of Mexico discharged 4.9 million barrels of crude oil from the Macondo well (OSAT/NOAA report, 2010). One of the first responses was to apply more than 1 million gallons of the oil dispersants Corexit 9527A® and Corexit 9500A® to the sea surface, and more than 770 thousand gallons to the sub-sea (On Scene Coordinator Report DWH, 2011). This large scale application of oil dispersants, motivated us to examine the effects of the dispersants on toxicity, especially given the limited toxicity information that is available (Judson et al., 2010).

Although oil dispersants are preapproved for this use and their deployment is widespread, there are doubts in the regulatory community about the efficacy of dispersants to ameliorate the biological impacts of oil spills because of the poor understanding of oil dispersant toxicity (Singer et al., 1998). Rigorous toxicological comparison of untreated and dispersant-treated oil is complicated by the fact that when oil, seawater, and dispersants are mixed, a complex multiphase system results. In this complex system, aquatic organisms can be exposed to many toxicants, in many forms, which can have several modes of action (National Research Council, 1989). Moreover, chemical dispersion of oil can yield: (1) dissolved petroleum hydrocarbons; (2) dissolved dispersant surfactants; (3) mixed droplets of bulk oil and surfactants (often in micellar form); and (4) nonmicellar, particulate bulk oil (Singer et al., 1998).

A second important issue for determining the effects of dispersants, is the separate and combined toxicity of the dispersant and the crude oil droplets. Toxicity became an important issue in the late 1960s and early 1970s when application of toxic products resulted in substantial loss of sea life (Fingas, 2002). Since that time, dispersants have been formulated to minimize toxicity to aquatic organisms. For example, the LC50 values of dispersants used in the early 1970s ranged from about 5 to 50 mg/L to the rainbow trout in 96 h exposures. In contrast, LC50s for dispersants available today vary from 200 to 500 mg/L and contain a mixture of surfactants and a less toxic solvent (Fingas, 2002). Nonetheless, use of oil dispersants remains a controversial countermeasure to minimize the impact of oil spills. Their ecological effects depend on whether oil dispersion increases or decreases exposure of aquatic species to toxic components of oil (Ramachandran et al., 2004). Ramachandran et al. (2004) evaluated whether fish exposure increased to polycyclic aromatic hydrocarbon (PAH) in dispersed oil relative to equivalent amounts of the water-accommodated fraction (WAF). They used fish cytochrome P4501A gene (CYP1A) induction in trout exposed to the dispersant Corexit 9500A, WAFs, and the chemically enhanced WAF dispersant of three crude oils. They concluded that Corexit 9500A® was not an inducer of CYP1A and it did not appear to affect the permeability of the gill surface to known inducers such as

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Table 1
Characteristics of the five strains of the *Brachionus plicatilis* species complex used in this work.

Description of strain	Abbreviation	Location of original collection	GenBank accession number of coxI gene sequence
Brachionus manjavacas	MAN	Sea of Azov, Russia	AY785194
Brachionus plicatilis sensu stricto	TOK	Tokyo, Japan	AY785175
Brachionus rotundiformis	HAW	Hawaii, USA	HM024708
Brachionus sp.	VER	Alvarado Lagoon, off the coast of Veracruz, Gulf of Mexico	JX644944

β-napthoflavone. Therefore, the use of oil dispersants will not increase the exposure of fish to hydrocarbons in crude oil.

The EPA required BP p.l.c. to use the *Brachionus plicatilis* acute toxicity test to assess the toxicity of oil dispersant mixtures in the Gulf of Mexico (U.S. EPA subsurface dispersant directive to BP, 2010). The species *B. plicatilis* has long been used in ecotoxicology to assess toxicity in marine waters (American Society for Testing Materials, 1998; Anon., 1998). It is one of the few cost-effective marine toxicity tests that can be replicated hundreds of times in a few days. *Brachionus plicatilis* was thought to be one species, and therefore only a single *Brachionus* marine species has been mostly used in toxicity tests, although at least 15 are believed to exist (Suatoni et al., 2006). Some of these may be more sensitive to toxicants or have other properties that make them more useful in toxicity assessments of marine waters. In light of the recent environmental catastrophe in the Gulf, it seemed prudent to systematically explore the full range of biodiversity of *Brachionus* species to identify the most sensitive species for marine toxicity assessment.

Therefore, the goals of our investigation are: 1) to study the effect of crude oil, Corexit 9500A® oil dispersant and its water-accommodated fractions on five *B. plicatilis* species complex lineages whose phylogenetic signature can be investigated and correlated with sensitivity to these toxicants, 2) assess the effects of a crude oil and Corexit 9500A® mixture at concentrations that are environmentally relevant.

2. Materials and methods

2.1. Sampling, resting egg hatching, and culturing

Geographical strains of marine *Brachionus* sp. were collected from 5 localities from several parts of the world (Table 1). The Veracruz strain is unable to produce cysts (at least under laboratory conditions) and therefore the culture was started from parthenogenetic females. Instant Ocean™ was use to prepare reconstituted seawater. Resting eggs of the other four strains were hatched in 15 psu reconstituted seawater approximately 15 cm below 40 W white fluorescent light bulbs. Rotifer were cultured in 3 mL in wells of a 9-well plastic plate filled with 15 psu reconstituted seawater, and the green alga *Tetraselmis suecica*.

2.2. Acute toxicity tests

We used the *B. plicatilis* acute toxicity test protocol described in Standard Methods (Anon., 1998) and in the American Society for Testing Materials (ASTM) protocol (ASTM, 1998). It is now understood that the species *B. manjavacas* is the species originally used to develop that protocol according to genetic analysis (Fontaneto et al., 2007). Instead of using neonates hatched from cysts (diapausing eggs) as described in protocol, we used neonates hatched from parthenogenetic eggs that were less than 24-h old. Toxicity tests with *Brachionus manjavacas* neonates hatched from cysts were also conducted to conform to the original Standard Methods and ASTM protocols and to compare results with neonates hatched from parthenogenetic eggs. A total of five independent replicates each consisting of 10 rotifer per well were conducted to obtain the Median Lethal Concentration (LC50) values for each treatment. The protocol for preparation of oil–water-accommodated-fractions (WAF) and enhanced water-accommodated-fractions (CEWAF) solutions for toxicity testing followed the recommendations of Singer et al. (2000). We stirred Macondo sweet crude oil with Instant Ocean® artificial seawater at 15 psu for 8-h with a magnetic stirrer to obtain the WAF's. LC50 values for crude oil, Corexit 9500A®, propylene glycol, which is a major component of Corexit 9500A® (Nalco Energy Services, 2012) and the Macondo oil fractions were calculated using probit models (Díaz et al., 2004).

2.3. Acute toxicity tests with Corexit 9500A:Macondo oil mix

Clark et al. (2001) suggest a 1:10 maximum exposure concentration for the Corexit 9500A®:oil mix. In contrast, the U.S. EPA (1995) recommended a 1:50 ratio. Therefore, we tested 1:10, 1:50 and 1:130 Corexit 9500A:Macondo oil ratios. This was accomplished by 8-h stirring of both the oil and the dispersant as previously described for preparing of WAF's. A different experiment consisted of adding 0.01% Corexit 9500A® (the 24-h NOEC value for Corexit with *B. manjavacas*) to a different set of Macondo oil concentrations to investigate synergistic effects during oil dispersion without stirring for 8-h. In this experiment the Corexit 9500A®:oil ratios were variable for each concentration ranging from 1:25 to 1: 500. Toxicity tests were done as described above. Five independent replicates each consisting of 10 rotifer per well were conducted to obtain the LC50 values for each treatment.

2.4. Reproductive and cyst hatching inhibition end-points

Reproductive tests were performed on neonates born from parthenogenetic *B. manjavacas* females according to the Standard Methods protocol (Anon., 1998). Twelve replicate neonates (five neonate rotifers per well), were exposed for 48 and 72-h to sublethal concentrations of Corexit 9500A® [1×10^{-6}–0.001% (v/v)], Macondo oil [0.25–5% oil (v/v)], and propylene glycol [0.1–5% (v/v)] in 1 mL volumes in a 24-well plate with 1×10^5 cells/mL of *Tetraselmis suecica*. The 24-well plates were then placed in a bioclimatic chamber under continuous light at a temperature of 25 °C for 48 and 72-h. At the end of these incubation periods, we counted the number of individuals in each well and calculated *r* (the instantaneous growth rate).

Cyst hatching inhibition assays consisted in hydrating dry *B. manjavacas* cysts for three hours, then, exposing them to same sublethal concentrations as above of Corexit 9500A®, Macondo oil WAF's, propylene glycol, or a Corexit 9500A®:Macondo oil mix for 24 or 48-h periods under fluorescent light. The number of hatching and non-hatching cysts was recorded, compared to controls in which no oil mixtures were added, in twelve replicates performed in three different dates. Each replicate consisted of ten cysts.

2.5. Statistical analysis and interpretation of data

We performed a one-way analysis of variance (ANOVA) with three independent treatments (each with four replicates) to compare five toxicant concentrations against the negative control and Dunnett's test to determine significant differences between the means of each toxicant concentration versus the no toxicity control. This allowed determination of the NOEC (no observed effect concentration) and the LOEC (lowest observed effect concentration). The EC50 values (the concentration where a 50% reduction in either the *r*-value or cyst hatching percentage, was observed) were calculated by linear regression of the different toxicant concentrations and the *r* values or cyst hatching percentages.

2.6. DNA sequencing

Genetic analyses using the cytochrome *c* oxidase subunit 1 (COI) gene were conducted to verify the species of each rotifer isolate used in these experiments. To minimize algal contamination, rotifers were incubated in 15 psu artificial seawater for 30 min to allow the rotifer guts to clear digested algal material. Genomic DNA was extracted from fresh rotifer tissue (500–1000 rotifers) using the DNeasy Tissue Extraction Kit (Qiagen). A 713 nucleotide region of the COI gene (Palumbi, 1996) was amplified via the polymerase chain reaction (PCR) using either universal COI primers LCOI1490: GGTCAACAAATCATAAAGATATTGG and HCOI2198: TAAACTTCAGGGTGACCAAAAAATCA (Folmer et al., 1994), or (VER strain only) degenerate COI primers modified from Folmer et al. (1994), dgLCO: GGTCAACAAATCATAAAGAYATYGG and dgHCO TAAACTTCAGGGTGACCAAARAAYCA (Meyer et al., 2005). Amplifications were performed in 10 μl volume solutions with 10–50 ng genomic DNA, 1 unit *Taq* DNA polymerase and a final concentration of 0.2 mM of each dNTP, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 2.5 mM $MgCl_2$, and 0.2 mM of each primer. Thermal cycling protocol conditions consisted of a denaturing step of 2 min at 95 °C followed by 40 cycles of 95 °C for 30 s, 47 °C for 90 s and 72 °C for 90 s on an Eppendorf MasterCycler. PCR products from TOK and HAW strains were directly sequenced in both directions (Nevada Genomics Center, University of Nevada, Reno). PCR products from the VER strain were cloned using TOPO TA Cloning Kit (Invitrogen) due to amplification with degenerate primers prior to sequencing. All sequences were manually edited in BioEdit vers 7.0.5.3 (Hall, 1999) and aligned using ClustalW (Larkin et al., 2007). Similarity to other *Brachionus* species was determined in a BLAST search (Altschul et al., 1990) of sequences deposited in the NCBI GenBank nucleotide database (www.ncbi.nlm.nih.gov).

Table 2
Comparison of lethal toxicity sensitivity among species of the *Brachionus plicatilis* species complex.

Species/strain	24-h LC50	LC50 95% confidence limits	NOEC	LOEC
Propylene glycol (mg/L)				
B. manjavacas from cyst	39.41	30.32–51.23	5.15	10.31
B. manjavacas parthenogenetic	26.50	19.32–36.32	25.90	51.80
B. plicatilis s.s. Tokyo strain	39.15	24.04–63.75	51.54	77.31
B. rotundiformis Haw strain	26.56	18.40–38.34	25.77	51.54
Brachionus sp. from Veracruz	31.47	19.05–51.32	10.31	25.77
Corexit 9500A® (mg/L)				
B. manjavacas from cyst	14.25	12.52–16.20	4.75	9.49
B. manjavacas parthenogenetic	10.39	8.69–12.42	9.49	14.24
B. plicatilis s.s. Tokyo strain	0.447	0.253–0.791	<0.474	0.474
B. rotundiformis Haw strain	1.75	0.98–3.12	0.47	0.95
Brachionus from Veracruz	4.30	3.38–5.48	1.19	3.56
Macondo sweet crude oil % water-accommodated fractions				
B. manjavacas from cyst	11.02	9.04–13.45	5.0	7.5
B. manjavacas parthenogenetic	5.43	3.98–7.42	7.5	10
B. plicatilis s.s. Tokyo strain	2.47	1.74–3.51	0.5	1.0
B. rotundiformis Haw strain	11.02	9.04–13.44	5.0	7.5
Brachionus sp. from Veracruz	19.33	14.65–25.49	10.0	12.5

2.7. Genetic analysis

To determine the relationship of the VER strain to other *Brachionus* species (Fig. 4), phylogenetic analysis of rotifers was assessed through neighbor-joining analysis of COI nucleotide sequences using PAUP* vers 4.0b10 (Swofford, 2002). Bootstrapping confidence values were determined over 1000 iterations. *B. calyciflorus* (GQ466414) was included as the outgroup based on this species' relationship to other brachionids in previous studies (Gómez et al., 2002; Suatoni et al., 2006).

3. Results

3.1. Acute toxicity test comparisons among species of the Brachionus plicatilis species complex

Acute toxicity of propylene glycol among the tested species of the *B. plicatilis* species complex ranged from LC50 = 26.50 mg/L *B. manjavacas* parthenogenetic to 39.4 mg/L for *B. manjavacas* hatched from cysts (Table 1). However, by comparing the 95% confidence limits, there were no significant differences among species in their acute toxicity response to propylene glycol.

The LC50s for Macondo oil acute toxicity ranged from 2.47 for *B. plicatilis sensu stricto* TOK strain to LC50 = 19.3 mg/L (*Brachionus* sp. VER strain). The decreasing sensitivities to Macondo oil were as follows: *B. plicatilis sensu stricto* > *B. manjavacas* parthenogenetic > *B. rotundiformis* = *B. manjavacas* from cysts = *Brachionus* sp. VER strain ($p < 0.05$ in all cases) (Table 2). The linear regression of the exposure concentration/response curve for Macondo oil for *B. manjavacas* hatched from cyst is shown in Fig. 1A.

For Corexit 9500A®, LC50s ranged from 0.447 for *B. plicatilis sensu stricto* TOK strain to 14.2 mg/L for *B. manjavacas* hatched from cysts (Fig. 1B). The decreasing Corexit 9500A® sensitivities were as follows: *B. plicatilis sensu stricto* > *B. rotundiformis* > *Brachionus* sp. VER strain > *B. manjavacas* parthenogenetic > *B. manjavacas* from cysts ($p < 0.05$ in all cases) (Table 2).

Acute toxicity of Macondo oil and Corexit 9500A® is similar in range (less than one order of magnitude) for most of the strains tested. However, Corexit 9500A® is consistently more toxic than propylene glycol. In the case of *B. manjavacas* there are no significant differences in the Corexit toxicity between females hatched from parthenogenetic eggs and females hatched from cysts. Likewise, there were few significant differences between parthenogenetic egg hatchlings and females hatched from cysts for Macondo oil ($p < 0.05$) (Table 2).

3.2. Reproductive and cyst hatching inhibition end-points

Reproductive tests were about 4-fold more sensitive than acute toxicity tests (Tables 2 and 3). For example, *B. manjavacas* hatched from cysts had a LC50 of 11.02% Macondo oil as compared to 2.55% EC50 for the reproductive test and the cyst hatching test. Both end-points follow similar dynamics, although for different time periods (Fig. 2). No significant differences ($p < 0.05$) were found when comparing the 48-h and 72-h EC50s of the reproductive test for both propylene glycol and Macondo oil (Table 3).

3.3. Synergistic effect of Corexit 9500A® and Macondo oil

Acute toxicity tests with 1:10, 1:50 Corexit 9500A®:Macondo oil mixtures resulted in 47–52-fold increases in toxicity (lower LC50 values in Table 4 and Fig. 3A and B). Similarly, addition of the 24-h NOEC concentration of Corexit 9500A® to Macondo oil increased toxicity by 27-fold (Fig. 3C). However, the 1:130 Corexit 9500A®:Macondo oil ratio mixture produced no increase in toxicity

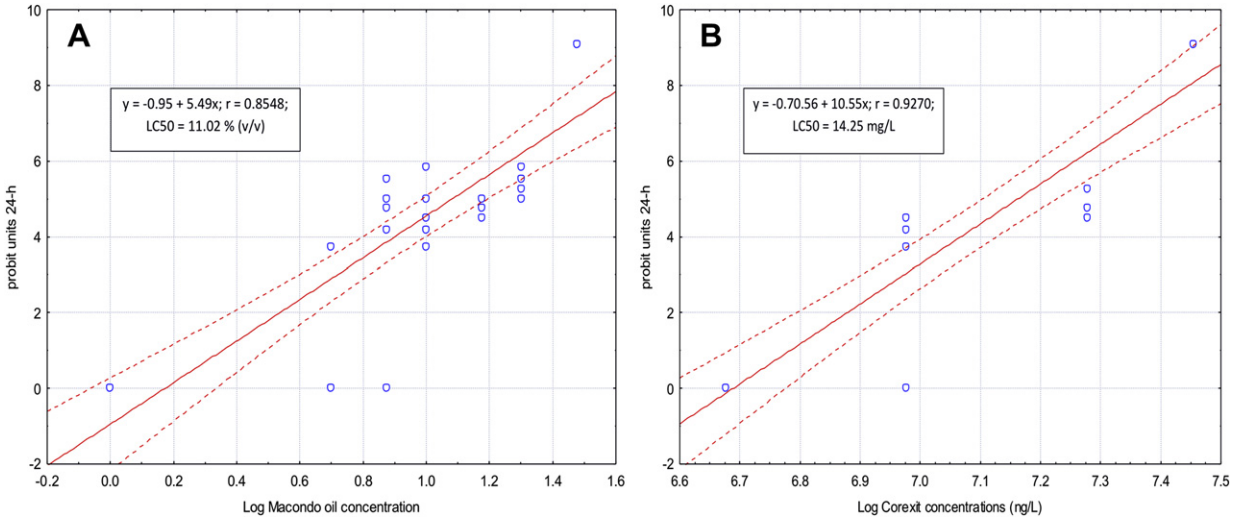

Fig. 1. Linear regression graphs of the results of the acute toxicity 24-h tests of *Brachionus manjavacas* neonates hatched from cysts exposed to: A) Macondo sweet crude oil. B) Corexit 9500A®. $N = 5$ for both treatments. Lines along the regression represent the 95% confidence intervals.

Table 3
Comparison of toxicant sensitivity among marine *Brachionus* species/strains. Reproductive and hatching inhibition end-points.

Species/strain	EC50	EC50 95% confidence limits	NOEC	LOEC
Propylene glycol (mg/L)				
Reproductive test				
B. manjavacas parthenogenetic	16.68 (48-h)	13.39–20.05	1.03	5.15
B. manjavacas parthenogenetic	19.03 (72-h)	14.24–20.80	5.15	10.31
Cyst hatching inhibition test (24-h)				
B. manjavacas	1.4194	1.2028–1.6359	1.2885	2.0616
Macondo sweet crude oil % water-accommodated fractions				
Reproductive test				
B. manjavacas parthenogenetic	2.50 (48-h)	2.03–2.97	1.0	2.5
B. manjavacas parthenogenetic	2.57 (72-h)	2.05–3.10	1.0	2.5
Cyst hatching inhibition test (24-h)				
B. manjavacas	2.55	1.70–3.39	1.0	2.5

(data not shown). The slope of the curve is steeper in Fig. 3A (1:10 ratio) and is reduced in Fig. 3B (1:50 ratio) and 3C (the NOEC-Corexit 9500A® experiment), as expected based on the toxicity of the 1:10 ratio > 1:50 ratio > NOEC-Corexit 9500A® treatments.

3.4. DNA sequencing and phylogeny analysis

COI sequences from MAN were confirmed to belong to *B. manjavacas* from GenBank (AY785194), TOK and HAW strains were consistent with sequences previously obtained from these strains and provided in GenBank (AY785175 and HM024708, respectively), confirming the TOK strain was *B. plicatilis sensu stricto* and the HAW strain was *B. rotundiformis* (Suatoni et al., 2006; Smith et al., 2011). The COI sequence for the VER strain was not previously represented in GenBank, but was most similar (*e*-value 3*e*-146 with 99% similarity) to two members (*B. ibericus s.s.* and *Brachionus* sp. Cayman clone) of the SM clade from the *B. plicatilis* complex (Gómez et al., 2002; Suatoni et al., 2006). The VER strain COI sequence was deposited in GenBank (JX644944). The COI phylogeny indicates a close relationship between the VER strain and a *B. ibericus* strain (okgu), supporting the hypothesis that the VER strain is a member of the SM clade from the *B. plicatilis* complex (Fig. 4).

Table 4
Synergistic effects of Corexit 9500A® when mix with Macondo oil ($n = 12$) with *Brachionus manjavacas* neonate hatchlings (less than 24-h old). $N = 5$.

Experiment description	LC50/95% CL	NOEC	LOEC	Increase in toxicity[a]
1:10 Corexit/Macondo oil Ratio (8-h stir)	0.21/0.17–0.27	0.05	0.10	52.48-fold
1.50 Corexit/Macondo oil Ratio (8-h stir)	0.23/0.19–0.28	0.05	0.10	47.91-fold
NOEC-Corexit/Macondo oil Ratio (no stir)	0.40/0.27–0.59	<0.25	0.25	27.55-fold

[a] Compare with *B. manjavacas* from cyst 24-h LC50 from Table 1.

4. Discussion

The 1:10, 1:50 and 1:130 ratios for the Corexit 9500A®:Macondo oil ratio mixture were chosen because they represent maximum exposure concentrations (Clark et al., 2001), or the recommended 1:10 to 1:50 ratios for Corexit application (U.S. EPA, 1995). The 1:130 ratio is the dispersant:oil mix actually used in the Deep Water Horizon spill: 4.9 millions of barrels of crude oil released into the Gulf of Mexico (OSAT/NOAA report, 2010), and about two million gallons of oil dispersant (mainly Corexit) applied to the Gulf of Mexico (On Scene Coordinator Report Deep Water Horizon, 2011). Corexit 9500A® and oil are more or less equivalent in toxicity (Fig. 1). However, when Corexit 9500A® and oil are mixed, our results show that Corexit 9500A®:Macondo oil at the recommended ratios increases acute toxicity up to 52-fold to *B. manjavacas*. Recall that this rotifer was endorsed by the EPA for this oil spill (U.S. EPA, Dispersant Monitoring and Assessment Final Directive for Subsurface Dispersant Application, 2010). Even if Corexit 9500A® was not mixed with oil for 8-h by stirring, its application increased toxicity of the dispersant:oil mixture by 27.6-fold. If we extrapolate the results of our experiments to the oil released by the Macondo well in the Gulf of Mexico, then the increase in toxicity by using Corexit, may have been markedly underestimated. What remains to be determined is whether the benefits of dispersing the oil by using Corexit 9500A® are outweighted by the substantial increase in toxicity of the oil: Corexit 9500A® mixture.

Surprisingly, there are few articles in the mainstream scientific literature recording synergistic effects of oil and oil dispersants resulting in toxicity increments (Mitchell and Holdway, 2000). Shafir et al. (2007) found that the dispersed oil and oil dispersants were more toxic to hard and soft coral species than crude oil, and

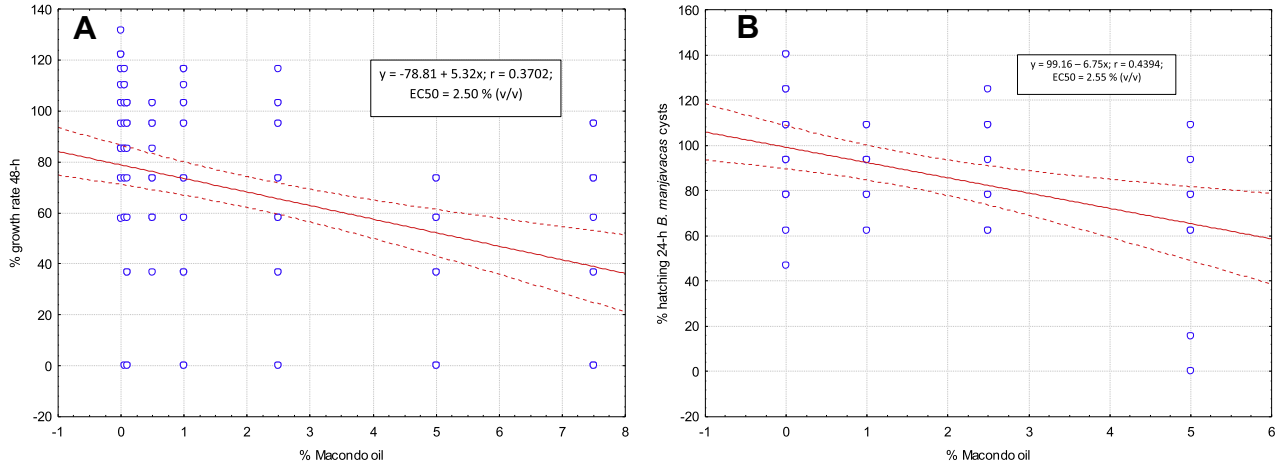

Fig. 2. Linear regression graphs of the results of the sublethal toxicity tests of *Brachionus manjavacas*. A) Reproductive test with neonates hatched from parthenogenetic eggs exposed 48-h to Macondo sweet crude oil. B) Cyst hatching inhibition 24-h exposure experiment.

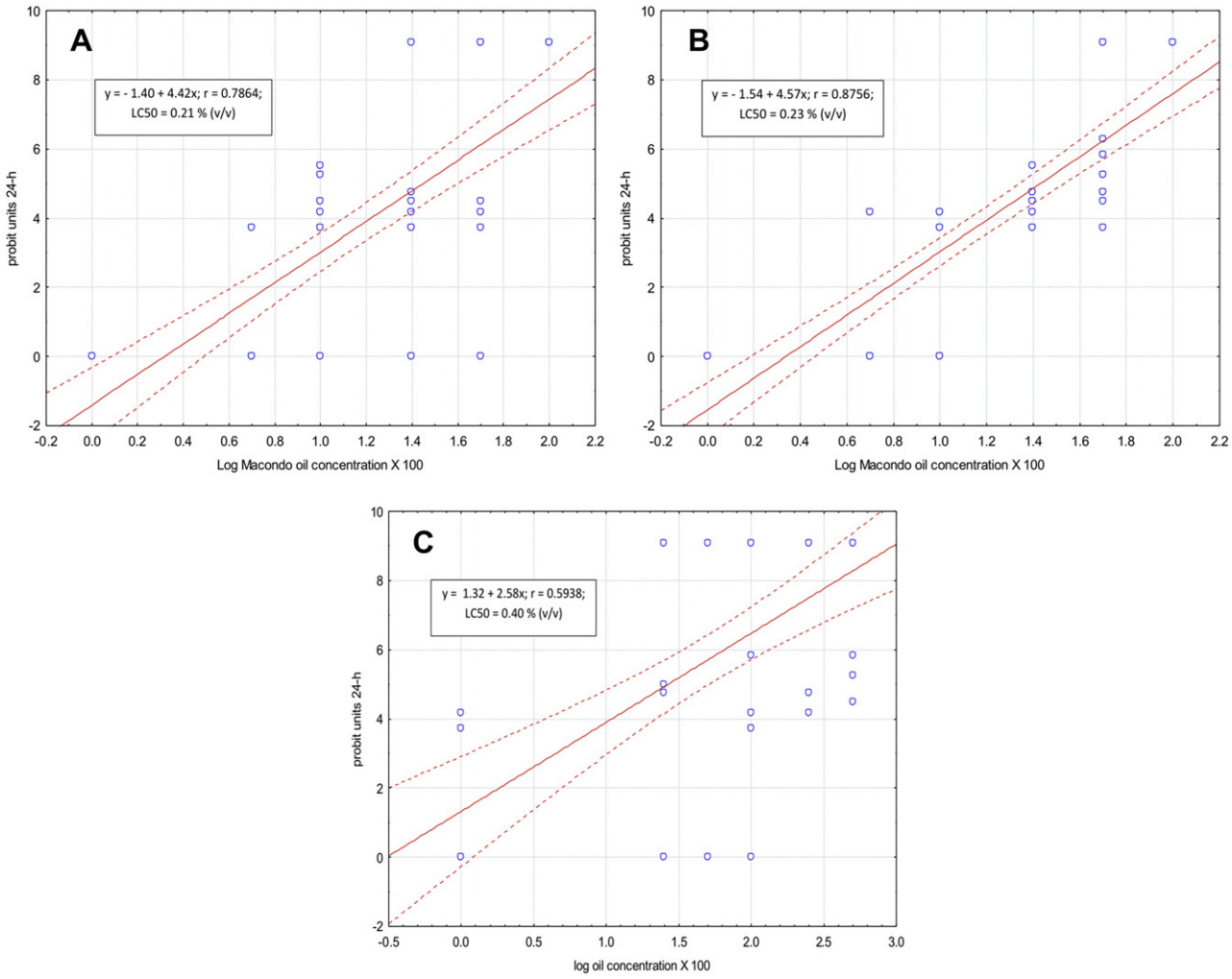

Fig. 3. Linear regression graphs of the results of the Corexit 9500A®:Macondo oil mixture experiments of *B. manjavacas* neonates hatched from cysts. A) 1:10 ratio with 8-h stirring prior to exposure. B) 1:50 ratio with 8-h stirring prior to exposure. C) Corexit 9500A®-NOEC added (0.01%) without 8-h stirring prior to exposure.

they recommend based on their findings, that no oil dispersant should be used near a coral reef. Milinkovitch et al. (2011) showed that juvenile, thin-lipped gray mullets (*Liza ramada*) exposed to oil dispersed chemically bioconcentrate more polycyclic aromatic hydrocarbons (PAH), and have higher mortality than mullets exposed to crude oil or oil that has been mechanically dispersed. Bhattacharyya et al. (2003) concluded that oil dispersants enhanced South Louisiana crude oil toxicity in microcosms containing three freshwater organisms. Hemmer et al. (2011) found that Corexit 9500A® has similar toxicity to other oil dispersants when mixed with South Louisiana sweet crude oil. Greer et al. (2012) performed wave tank experiments that demonstrated that Corexit 9500A® increase the toxicity of petroleum to herring embryos by increasing the amount of petroleum hydrocarbons in the water column. Wu et al. (2012) found that Corexit 9500A® enhanced toxicity by 30–360 times in terms of percentage of v/v because dispersion by Corexit 9500A® accelerated partitioning of hydrocarbons making them more bioavailable to rainbow trout embryos.

An interesting result is that the *Brachionus* sp. VER strain from the Gulf of Mexico was the most tolerant to Macondo oil (LC50 = 19.33% in Table 2). Perhaps, *Brachionus* sp. VER strain adapted to petroleum from natural seeps in the Gulf of Mexico. However, this explanation deserves further investigation.

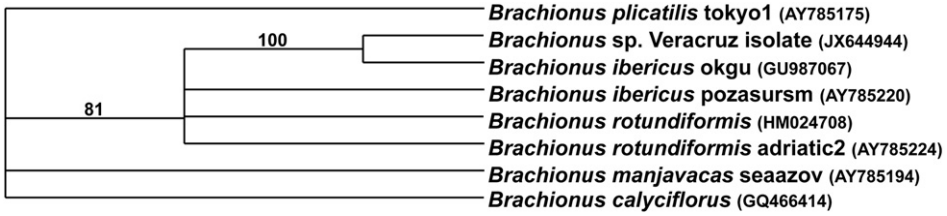

Fig. 4. Phylogenetic reconstruction of *Brachionus* rotifers using neighbor-joining analysis based on the cytochrome *c* oxidase subunit 1 gene. *B. calyciflorus* was included as the outgroup. Numbers on branches indicate support (≥70%) after 1000 bootstrap replicates. GenBank accession numbers are provided after species names.

We confirmed the identity of each of the four *Brachionus* species used in the toxicant sensitivity experiments through genetic analysis of the mitochondrial COI gene (Fig. 4), and allows confirmation that differences in sensitivity among species of the *B. plicatilis* species complex are minor for the three toxicants tested, mostly within one order of magnitude. This likely reflects the phylogenetic closeness of these sibling species (Table 2). This may be important since the RotoxkitM™ (Microbiosystems. http://www.microbiotests.be), the commercial toxicity kit suggested by EPA to monitor Macondo oil toxicity (U.S. EPA, 2010), contains cysts of *Brachionus manjavacas* although it is labeled as *Brachionus plicatilis*. This is because the kit was developed when *Brachionus plicatilis* was recognized as a single species rather than a species complex. Further studies by several authors (see Segers, 1995; Ciros-Pérez et al., 2001; Suatoni et al., 2006; Fontaneto et al., 2007) have shown that the original lineage used to produce the RotoxkitM™ cysts is indeed *Brachionus manjavacas*, based upon the genetic signature of the COI sequence, and is well recognized today.

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